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Commentary

Why Do We Expect Carotenoids to be Antioxidants *in vivo?*

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The antioxidant properties of β-carotene, in addition to its proposed immunomodulatory effects, have often been cited as the factors underlying its role in preventing disease initiation and propagation, yet the strongest evidence for diet and cancer prevention is based on fruit and vegetable intake and not β-carotene or other dietary carotenoids, per se. In the light of the outcome of the ATBC trial, the Physicians Health Study and the premature termination of the CARET study, this review addresses the issue of the antioxidant properties of the carotenoids and poses the questions: do dietary carotenes and xanthophylls have a clear role in disease prevention and are their antioxidant properties relevant to this role? What do we know about their mechanisms of action *in vitro* as free radical scavengers?

keywords: Carotenoid, β-carotene, xanthophyll, lycopene, $ABTS^{\bullet+}$, electron-donor, radical scavenger, antioxidant

INTRODUCTION

Numerous epidemiological studies have shown a consistent inverse relationship between dietary intake of carotenoid-rich foods and the incidence of lung cancer and also, though less so, with cancers of the mouth, pharynx, oesophagus, stomach, colon, rectum, bladder and cervix; this is as well as associations with coronary heart disease, cataract and macular degeneration.[1–5] The assumption has been made that important factors contributing to disease protection include the antioxidant properties of a number of constituents, including the carotenoids. However, a number of recent intervention studies involving supplementary β-carotene have questioned the role of carotenoids: two cases, the α-Tocopherol β-Carotene Cancer Prevention Trial (ATBC)[6] and the Carotene and Retinol Efficacy Trial (CARET),[7] suggested an adverse effect on lung cancer, while the outcome of the Physicians' Health Study was that long-term supplementation with β-carotene had no effect on the incidence of malignant neoplasms and cardiovascular disease.[8]

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These findings raise a number of issues, particularly since a clear, inverse relationship between fruit and vegetable intake and cancer incidence has been demonstrated in many epidemiological studies worldwide. In at least 15 of these, blood levels of β-carotene were significantly elevated in the group taking the larger amounts of fruit and vegetables. However, the strongest evidence concerning diet and cancer prevention is based on fruit and vegetable intake, not on β-carotene or other carotenoids *per se*. Thus, many other constituents of fruit and vegetables may contribute to the anticarcinogenic properties, including: vitamin C, vitamin E, selenium, flavonoids, phenolics, phyto-oestrogens, allium compounds, limonene, glucosinolates and indoles, dithiolthiones, isothiocyanates, protease inhibitors, fibre and folic acid; these, either independently or in combination, might act as anti-cancer agents by a variety of mechanisms.

Due to their wide range of biological properties,[9] it is obvious that carotenoids can exert bioprotective effects through a variety of mechanisms. For instance, β-carotene is particularly effective as a photoprotective agent when administered to patients suffering from photosensitivity diseases such as erythropoietic protoporphyria.[10] The same compound is capable of restoring cell-to-cell communication between cancer cells *in vitro*[11] and has significant immunomodulatory properties.[12] Some dietary carotenoids are also precursors of a separate class of bioactive compounds, the retinoids, which can regulate cell growth and differentiation in various cell types through interaction with ligand-dependent transcription factors.[13] But it is the antioxidant properties of β-carotene,[14] in combination with its immunomodulating properties, which have most often been the focus of its role in preventing disease initiation and propagation. Lower serum β-carotene levels have been linked to higher rates of cancer and cardiovascular disease, as well as increased risk of myocardial infarction among smokers. Blood levels of carotenoids may simply reflect fruit and vegetable consumption—other candidate constituents which may act in concert with or independently of the carotenoids have not been evaluated. In the light of the results from the ATBC trial,[6] the Physicians' Health Study,[8] as well as the premature termination of the CARET study,[7] a number of questions are posed: do carotenoids have a clear role in disease prevention? Are the antioxidant properties of carotenoids relevant to this role? What do we know about the mechanisms of action of carotenoids *in vitro* as free radical scavengers?

STRUCTURAL FEATURES OF CAROTENOIDS

The carotenoids are the most widespread group of pigments in nature, with more than 600 different naturally-occurring structures identified. They are based upon the same C_{40} isoprenoid skeleton, which is modified by cyclisation, addition, elimination, rearrangement and substitution. The carotenoid hydrocarbons are collectively known as the carotenes, and are typified by the acyclic lycopene and bicyclic β-carotene (Fig. 1). The oxygen-containing carotenoids are called the xanthophylls, *eg.* lutein and canthaxanthin (Fig. 1). [The vast array of carotenoid structures can be found in *Key to Carotenoids*.[15]] Traditionally, carotenoids have been given trivial names as used in this article, but a semi-systematic nomenclature, conveying structural information, has been devised.[16]

The carotenoids owe their characteristic colours to the absorption of light (typically 400–500 nm wavelengths) by a chromophore of conjugated double bonds. In lycopene and β-carotene (Fig. 1) this is entirely made up of carbon-carbon double bonds but in some molecules, such as canthaxanthin, two carbon-oxygen double bonds extend the polyene chain chromophore. In principle, each of the polyene chain double bonds could exist in a *cis* or *trans* conformation, thus creating an enormous number of geometric isomers. In practice, however, few isomers exist. The reason for this is that the introduction of a *cis* double bond results in steric

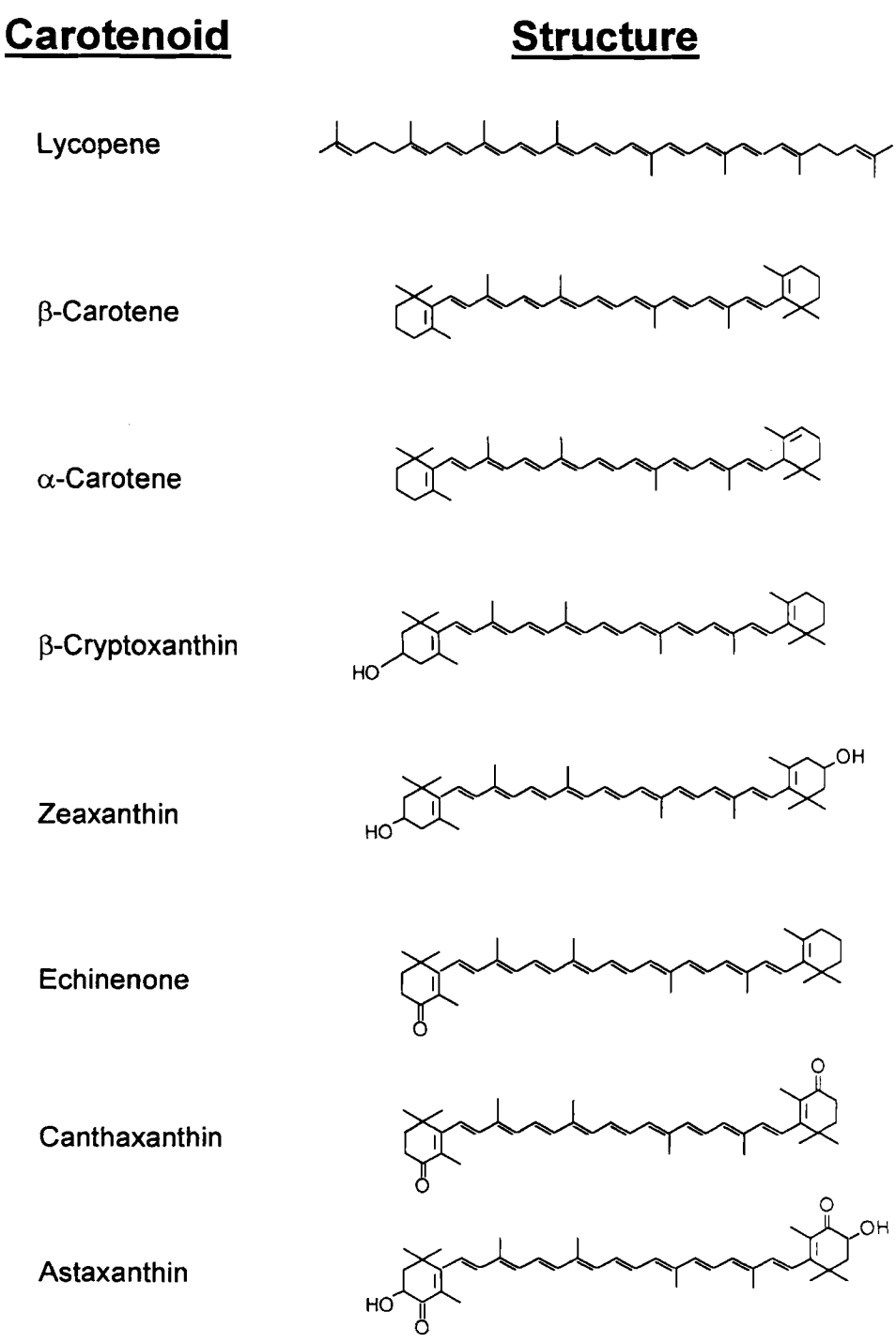

FIGURE 1 Structures of the common carotenoids.

hindrance, making that isomer less stable than the *trans* form. Thus, the vast majority of carotenoids are in the all-*trans* configuration, as shown in Figure 1. The few examples of *cis* isomers occur when the steric hindrance in the molecule is minimal, *eg.* 9-*cis*-β-carotene.

Along the backbone of the carotenoid molecule the polyene chain contains delocalised π-electrons which are responsible for the electronic spectra of the carotenoids (reviewed in [17]). As the chromophore is extended, the π-electrons can be more easily transferred to the π* excited state, which can be sufficiently low in energy in such a polyene chain to correspond to absorption of light in the visible region. The electron density across the chromophore is not uniform, however, and is greater at the ends. X-Ray crystallography shows that the carotenoid molecule has a slight S-shaped distortion to relieve steric tension across the polyene chain.[18]

Most carotenoids have two cyclic end groups, typically the β- or ε-type. β-Carotene contains two β-groups, whilst α-carotene has one β- and one ε-ring (Fig. 1). The C-6,7 and C-6′,7′ single bonds in such molecules could lead to an infinite twisting of these rings relative to the polyene chain. In the β-ring, the C-5,6 double bond is conjugated to the polyene chain, so that coplanarity should occur. It is known that the 6-s-*cis* conformation is preferred, as shown in β-carotene, but a distortion of some 40° is found to relieve steric hindrance between the C-5 methyl group and the C-8 hydrogen atom.[19] Orbital overlap with the polyene backbone is therefore reduced and the contribution of the ring carbon-carbon double bonds to the chromophore is small. No such conjugation exists with the ε-ring, as in α-carotene, so that steric hindrance is the only factor determining the preferred conformation. The rings themselves are normally chair or half-chair in shape, thus producing bulky end groups in contrast to the long, linear acyclic carotenoid shape typified by lycopene. Functional groups, *eg.* epoxides, hydroxides and carbonyls may also be present on these rings, adding to their steric bulk and chemical reactivity.

The polyene chain is highly rich in electrons and, therefore, susceptible to electrophilic attack. Thus, oxidising agents and free radicals react rapidly with carotenoids *in vitro*. Indeed, carotenoids in solution are easily degraded in the presence of only traces of oxygen, leading to a loss of colour as the chromophore is cleaved. *In vivo*, however, the carotenoid molecule is often stabilised against electrophilic attack by proteins, especially lipoproteins and lipid/protein regions of membranes, due to their hydrophobic properties. Therefore, the instability of a carotenoid in solution may not reflect its rate of degradation in the cell. In addition, the orientation of the carotenoid within a membrane may influence its susceptibility to different types of free radical attack. If the molecule is embedded within the inner, hydrophobic areas of a membrane, then it will only react efficiently with those radicals located in the same milieu. In contrast, polar functional groups of the xanthophylls such as hydroxyls render them more accessible to the aqueous environment.[17]

ANTIOXIDANT PROPERTIES

An antioxidant has been defined[20] as a substance which, when present at low concentrations relative to the oxidizable substrate, can suppress, delay or prevent oxidation of that substrate. The activity of an antioxidant is determined by:

(a) its chemical reactivity as an electron-donor or hydrogen-donor in reducing the free radical;
(b) the fate of the resulting antioxidant-derived radical and its ability to stabilize and delocalize the unpaired electron through the conjugated double-bonded system;
(c) its reactivity with other antioxidants present;
(d) its reactivity with molecular oxygen.

Thus, the antioxidant activity of β-carotene and the other carotenoids will not only reflect the rates of free radical scavenging but also the reactivity of the resultant β-carotene-derived radi-

cals, *ie.* the stability of the resonance stabilised carbon-centered carotenyl radical formed.

The ability of the antioxidant radical to delocalise the unpaired electron, and hence prevent its reactivity as a free radical, is fundamental to its properties as an antioxidant. Another important factor contributing to the antioxidant properties of carotenoids and their rates of consumption is their reactivity with oxygen. It has long-been demonstrated that β-carotene is more efficacious as an antioxidant under low oxygen tension,[21] and this observation exemplifies the relevance of interactions with molecular oxygen to the antioxidant activity of the carotenoids.

The mode of action of carotenoids as antioxidants has been linked to their ability to quench singlet oxygen[22] and prevent lipid peroxidation *in vitro* caused by singlet oxygen.[23–26] In addition to quenching singlet oxygen, carotenoids can intercept the propagation step of lipid peroxidation *in vitro*.[14,27] The resulting carbon-centered radical is stabilised by the presence of the conjugated double bond system which facilitates a resonance condition.[21]

The structural properties of the carotenoid, in particular the length of the polyene chain, can significantly influence its antioxidant properties.[27,28] An essential aim has been to determine which factors define these differences in antioxidant activity. What is the influence of the β-ionone ring on the antioxidant activity of β-carotene compared, for example, with the activity of lycopene, which lacks the ring? How does addition of a substituent such as a carbonyl or hydroxyl group to the β-ionone ring (which increases the polarity of the carotenoid), or modification of the hydrocarbon polyene chain, affect the quenching ability? The following section describes studies that have approached these issues.

I Mechanistic Studies

Recent studies employing pulse radiolysis and rapid, time-resolved spectrophotometry have shown that carotenoids react with oxidising radicals by a variety of mechanisms.[29–34] To date, the initial products obtained when carotenoids react with these radicals (Fig. 2) indicate that the kinetically favoured reactions are electron transfer, whereby the carotenoid (Car) is oxidised to its radical cation ($Car^{\bullet+}$; Reaction 1),

$$Car \rightarrow Car^{\bullet+} + e^- \qquad [1]$$

and radical addition, giving rise to carotenyl adduct radicals such as $[R\text{-}Car]^{\bullet}$ (Reaction 2).

$$Car + R^{\bullet} \rightarrow [R\text{-}Car]^{\bullet} \qquad [2]$$

In general, the rates of these reactions compare favourably with the corresponding reactions between the same oxidants and polyunsaturated fatty acids, indicating that carotenoids possess the required reactivity to function as antioxidants. The mechanisms of electron transfer pathways and radical-addition pathways are described in more detail below.

(1) **Electron transfer pathways** form the radical cation which is easily identified from its characteristic absorbance in the near infra-red.[35,36] The nitrogen dioxide radical, $NO_2^{\bullet}$ (relevant to cigarette smoke, which has been shown to destroy carotenoids in plasma),[37] the trichloromethyl radical, $CCl_3^{\bullet}$, and the bromine radical anion, $Br_2^{\bullet-}$, are all reduced by β-carotene through a mechanism of electron donation (Reaction 3).

$$NO_2^{\bullet} + Car \rightarrow Car^{\bullet+} + NO_2^- \qquad [3]$$

Electron paramagnetic resonance measurements of several carotenoid radical cations indicate a polyene π-radical structure with the unpaired spin extensively delocalised throughout the polyene chain and little unpaired electron density occurring near the terminal groups.[35,38–40] Such carotenoid radical cations decay slowly by self-reaction, the kinetics of which allude to a bimolecular process[33] (Reaction 4).

$$2Car^{\bullet+} \rightleftharpoons Car + Car^{2+} \qquad [4]$$

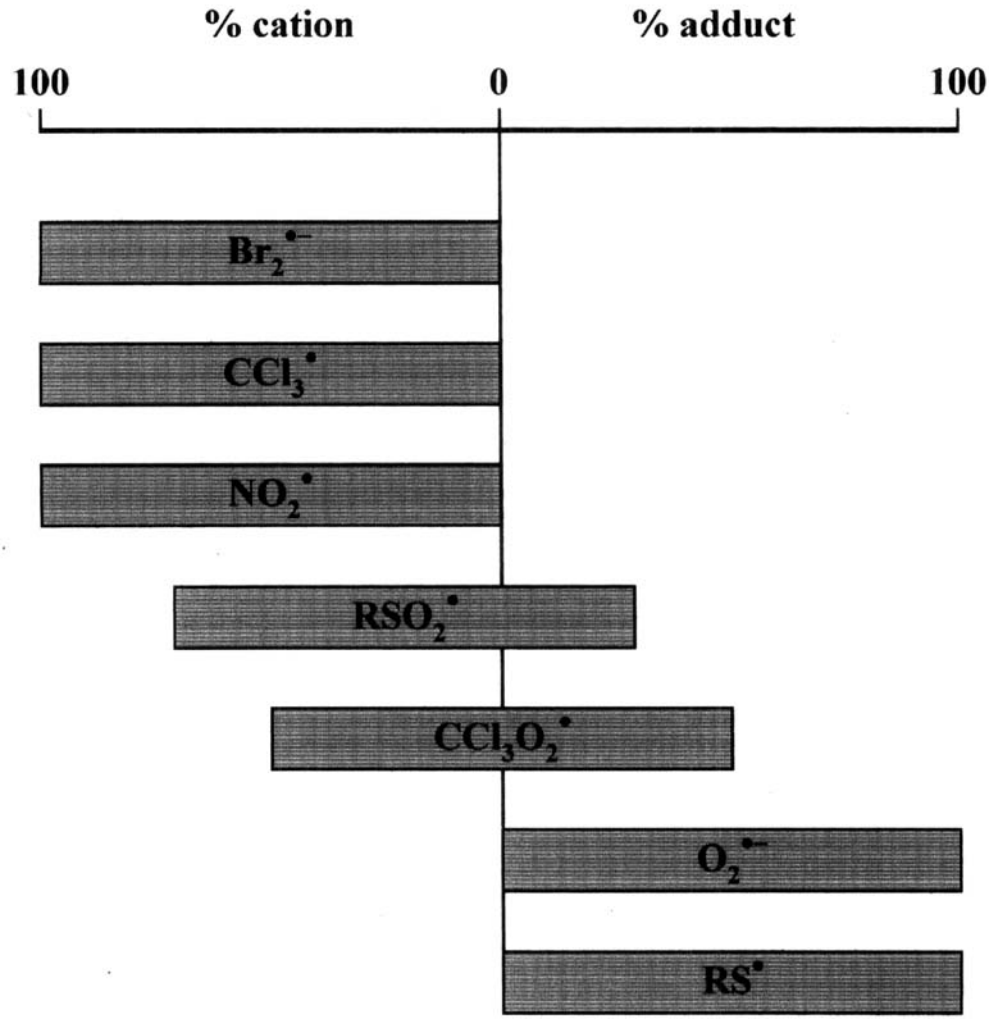

FIGURE 2 Reaction between β-carotene and various oxidizing radicals—distribution of initial products. Summary of the results from several studies[29–34] investigating the mechanism of reaction between β-carotene and oxidising radicals. In each case, the listed radical was generated by pulse radiolysis and the product distribution was estimated by spectrophotometry.

As Reaction 4 indicates, the $Car^{\bullet+}$, Car and Car^{2+} species in fact exist in a comproportionation equilibrium.[40] The position of this equilibrium and the lifetime of several $Car^{\bullet+}$ species have been assessed by direct measurement of the concentration of $Car^{\bullet+}$ and Car^{2+} formed electrochemically in solution.[36,40] It was found that the greater the number of keto groups present at the end of the chromophore, the more the equilibrium favoured $Car^{\bullet+}$. Canthaxanthin$^{\bullet+}$ was longer-lived than β-carotene$^{\bullet+}$. Further channels of decay have necessarily been invoked to account for the disappearance of Car^{2+} at ambient temperature,[36,38,41] but these have not been well-characterised. Hence, the significance of the comproportionation equilibrium with regard to antioxidant function is at present unclear.

The energy required to drive Reaction 1 forward is related to the standard one-electron reduction potential (E^{o}_{1}) of the $Car^{\bullet+}$ species. By using cyclic voltammetry under convenient (*ie.* non-standard) conditions, estimation of some

form of reduction potential (E_1) has now been achieved for a number of carotenoids[35,38,42,43] (Table I). The need to conduct these measurements in an aprotic organic solvent such as dichloromethane makes their comparison with other (usually aqueous) measurements troublesome, but on consideration of the general magnitudes of these potentials, the transfer of an electron from carotenoid to many oxidants of physiological importance[44] is undoubtedly thermodynamically feasible.

The spread of E_1 values in Table I suggest that there may be significant differences in the reactivities of diverse carotenoids with one-electron oxidants. For example, addition of a keto group to the 4-position of β-carotene to form echinenone results in a 60 mV increase in E_1, and additional modification of the 4'-position to form canthaxanthin increases E_1 a further 115 mV.[38] Other electrochemical studies with synthetic carotenoids confirm that, overall, carotenoids substituted with electron-donating groups are more easily oxidised than those with electron-accepting substituents.[39]

(2) **Radical-addition pathways** are involved where lipid peroxyl radicals[21] and thiyl radicals,[33] such as those derived from glutathione, interact with β-carotene by addition of the free radical to the conjugated π-electron system of the carotenoid (Reactions 5 and 6). The carbon-centred adduct radical from the thiyl radicals is deemed to be relatively unreactive due to resonance stabilization. Oxygenation of the adduct is possible, however, forming a carotenoid peroxyl radical which is capable of hydrogen abstraction.[32]

$$ROO^{\bullet} + Car \rightarrow [ROO - Car]^{\bullet} \qquad [5]$$

$$RS^{\bullet} + Car \rightarrow \underbrace{[RS - Car]^{\bullet}}_{\text{carbon-centered radical adduct}} \xrightarrow[\text{possible}]{+O_2} RS - Car - OO^{\bullet} \qquad [6]$$

A variety of decay routes have been observed for carotenoid-adduct radicals. For instance, whilst [CCl_3OO-Car]$^{\bullet}$ adducts decayed to leave the carotenoid radical cation,[34] [RS-Car]$^{\bullet}$ adducts underwent bimolecular decay.[33] It is essential to consider the possibility of secondary reactions between carotenoid radical adducts and molecular oxygen to give peroxyl radicals (Reaction 7).

$$[R - Car]^{\bullet} + O_2 \rightarrow R - Car - OO^{\bullet} \qquad [7]$$

Since any R-Car-OO$^{\bullet}$ radical could have significant chain-carrying pro-oxidant character, this reaction is of central importance to the notion that carotenoids could function efficiently as adduct-forming antioxidants. For this notion to be justified, conversion of [R-Car]$^{\bullet}$ to non-radical products needs to compete favourably with any reaction between [R-Car]$^{\bullet}$ and oxygen *in vivo*. The ability of high oxygen tensions (>150 Torr) to impart pro-oxidant character to β-carotene in peroxidising lipid systems *in vitro*[21,45] is strong evidence that carotenoid peroxyl radicals are indeed reactive and could initiate damage *in vivo*. At the same time, the protective effect of β-carotene observed at low oxygen tensions (≤150 Torr) supports the argument that, in the absence of oxygen, carbon-centred carotenyl radicals are relatively unreactive species. By way of comparison, the equivalent reaction between Car$^{\bullet+}$ and molecular oxygen is not apparent.[46]

TABLE I Formal reduction potentials for carotenoid radical cations

Carotenoid radical cation	E_1 (mV) vs S.C.E.	Reference
β-carotene$^{\bullet+}$	530	38
	720	42
	780	35
	742	43
echinenone$^{\bullet+}$	590	38
canthaxanthin$^{\bullet+}$	705	38
	900	35
	882	43
β-*apo*-8'-carotenal$^{\bullet+}$	910	35
	910	43

Compilation of formal reduction potentials (E_1) for the couple Car$^{\bullet+}$ + e^- → Car, each estimated by cyclic voltammetry in dichloromethane. E_1 is quoted *versus* a KCl-saturated calomel electrode (S.C.E.), the reference electrode used in the majority of these studies.

Molecular (as opposed to radical) adducts produced by the reaction of carotenoids with free radicals seem to be unstable towards analytical procedures such as HPLC and HPLC-MS, and have consequently eluded detection by these techniques. But recently, using atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and collision induced dissociation mass spectrometry (CID-MS), Liebler and McClure have detected molecular adducts formed between β-carotene and the alkyl and alkoxyl radicals produced by the decomposition of the azo compound AMVN.[47] Interestingly, the products identified imply that, in addition to being able to trap AMVN-derived radicals by direct addition, β-carotene can act as a hydrogen donor, at least in non-polar solvents. This possibility was first suggested some time ago.[48] Adducts formed between β-carotene and AMVN-derived peroxyl radicals were too short-lived to be detected by the APCI-MS procedure, but β-carotene epoxides believed to arise from their decomposition were in evidence.

(3) **A combination of the two pathways** has also been demonstrated, for example, on interaction of β-carotene with sulphonyl radicals (Reaction 8) and with phenoxyl radicals (which are formed on scavenging of free radicals by phenolic antioxidants). In the case of sulphonyl radicals, the distribution of products is approximately 3 parts radical cation to 1 part adduct radical.[33,49]

$$RS^{\bullet} + O_2 \rightarrow \underset{\text{sulphonyl radical}}{RSO_2^{\bullet}} \xrightarrow{+Car} \underset{3 \text{ radical cation}}{Car^{\bullet+}} : \underset{1 \text{ adduct radical}}{+[RSO_2 - Car]^{\bullet}}$$

Both form highly resonance stabilised structures and undergo slow bimolecular decay to non - radical products [8]

Likewise, when β-carotene reacts with the phenoxyl radical (generated by flash photolysis), it is possible to measure the kinetics of the parallel processes occurring, namely electron transfer from β-carotene to the phenoxyl radical and formation of an adduct between the two.[50] Upon challenging β-carotene with $CCl_3O_2^{\bullet}$, there is fairly even competition between electron transfer and radical addition reactions.[34]

Addition of substituents to the β-carotene skeleton can also influence the molecule's propensity to react by either of these pathways. For example, upon reaction with $CCl_3O_2^{\bullet}$, β-carotene and canthaxanthin yield both cation and addition radicals as initial products, whereas astaxanthin yields only addition radicals.[34] In a similar vein, β-carotene forms an adduct with $O_2^{\bullet-}$ but lycopene undergoes electron transfer.[30,32] The relative proportions of the two carotenyl radicals formed may reflect the free energy changes, or perhaps simply the activation energies of the competing processes. The latter is a possibility if, as has been suggested,[21,48,51] the most reactive positions are located near the chromophore termini. Both ring formation and multiple substitution significantly change the geometry and accessibility of these regions, and one can imagine how steric barriers to the formation of specific transition states may play a part in determining the rates of these reactions.

II Inhibition of Lipid Hydroperoxide Formation

The relative abilities of carotenes and xanthophylls to inhibit lipid hydroperoxide formation, induced by azo initiators in homogeneous lipid systems, are consistent across many studies. These studies have frequently been interpreted using the assumption that the reactions between the azo initiator-derived radicals and the polyunsaturated fatty acids are much faster than those between the same azo initiator-derived radicals and the carotenoids under investigation. This assumption is as yet untested, so the contribution of the latter process to the observed protective effects remains unquantified.

Astaxanthin and canthaxathin are more effective antioxidants than β-carotene or zeaxanthin in retarding hydroperoxide formation on azo-

initiated lipid peroxidation in homogeneous methyl linoleate/AMVN systems, and yet the rates of AMVN-induced oxidation of astaxanthin and canthaxanthin are slower than those of β-carotene and zeaxanthin.[27] The question might be posed as to precisely how the presence of these carbonyl groups increases the effectiveness of carotenoids in suppressing hydroperoxide formation; Terao[27] proposed that substitution of the hydrogens with carbonyl groups at the 4- and 4'-positions increases the overall peroxyl radical trapping efficacy (*astaxanthin* ≈ *canthaxanthin* >> β-*carotene* ≈ *zeaxanthin*) by virtue of the fact that the electron-withdrawing character of the carbonyl oxygen atoms substantially reduces the unpaired electron density throughout the carbon skeleton, decreasing the reactivity of the carbon-centred radical towards oxygen. These observations are supported by those of Jørgensen and Skibsted[52] in their ranking of the antioxidant activities of *astaxanthin* > *canthaxanthin* > β-*carotene* > *zeaxanthin* in a homogeneous methyl linoleate/AIBN system, consistent with the idea that the keto carotenoids are more effective antioxidants against lipid peroxidation *in vitro*. From this study, the implication again is that astaxanthin and canthaxanthin are more effective than β-carotene and zeaxanthin in stabilising *trapped* peroxyl radicals.

Studies employing micellar or liposomal systems offer a more complex picture. For example, when peroxidation of methyl linoleate/Tween 20 emulsions was promoted *in vitro* by haem proteins (metmyoglobin), all the carotenoids studied protected against lipid hydroperoxide formation but with no dependence on carotenoid structure.[52] Yet, when incorporated into linoleic acid/SDS micelles, β-carotene was ineffective against AAPH-initiated peroxidation.[53] In further contrast, carotenoids incorporated into phosphatidylcholine liposomes did protect them against AMVN-initiated peroxidation, with a ranking of effectiveness similar to that observed in homogeneous systems: *astaxanthin* > *zeaxanthin* > *canthaxanthin* >> β-*carotene*.[28] But, when peroxidation of similar liposomes was initiated by water-soluble AAPH, the order of effectiveness changed to *astaxanthin* ≈ *zeaxanthin* >> β-*carotene* > *canthaxanthin*, suggesting that when using this type of initiator, it is the presence on each ring of hydroxyl groups, rather than conjugated keto groups, which imparts increased antioxidant activity.

These types of heterogeneous system, which can be extended to include the LDL particle, raise the issue of carotenoid localisation within ordered lipid structures and what effect this may have upon their effectiveness against lipid peroxidation initiated by various means. It is known that the structure of a particular carotenoid has a significant bearing on its location and preferred orientation within phospholipid bilayers.[17] Hydrocarbons, such as β-carotene and lycopene, are located entirely within the hydrophobic membrane core and display some disorder in their orientation, the degree of which depends on their concentration and the temperature.[54–57] In contrast, xanthophylls such as zeaxanthin which contain two distal polar groups adopt a rigid, membrane-spanning orientation and have been likened to molecular rivets.[58] Such an orientation may confer the ability to trap radicals across most of the bilayer thickness. Extrapolating these observations to the ordered (but distinctly different) structure of the LDL particle, one expects carotenes to be found solely in the cholesterol ester-rich core, a location which enables them to interact efficiently only with the non-polar radicals which are formed in, or diffuse into, this compartment. Carotenoids containing polar groups may well have greater access to radicals entering and propagating at the LDL periphery, and may consequently be more effective protectants against some pro-oxidant stimuli.

III Ranking of Reactivity with Free Radicals

The direct interaction of carotenoids with free radicals has shown that:

(i) singlet oxygen has a greater reactivity with *lycopene* > β-*carotene*.[26,59]

(ii) interaction with the the stable radical cation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate), $ABTS^{\bullet+}$,[60] demonstrates a ranking of reactivity of carotenoids as antioxidants, related to their reactivities with one-electron oxidants:

$$lycopene > \beta - carotene = \beta - cryptoxanthin > \underset{-3,3'-diol}{lutein / zeaxanthin} > \underset{-4-one}{echinenone} > \underset{-4,4'-dione}{canthaxanthin} = \underset{-3,3'-dihydroxy-4,4'-dione}{astaxanthin}$$

This chromogenic redox-indicator[61] was used to identify those carotenoids that most readily undergo one-electron oxidation. Based on its ability to quench the coloured $ABTS^{\bullet+}$ species within a fixed time period, each carotenoid was assigned a Trolox-equivalent antioxidant capacity (TEAC; Table II). The activity of Trolox, the α-tocopherol analogue, was given a value of 1; any compound with a higher value indicates a higher activity in the assay, when compared on a molar basis. A wide range of activities was observed. Amongst the β-carotene analogues, the relative activities of β-carotene, echinenone and canthaxanthin are found to be in accordance with the relative reduction potentials of their radical cations, *ie.* β-*carotene* > *echinenone* > *canthaxanthin* by TEAC. Canthaxanthin and astaxanthin were ineffective reductants in this system, a property which may be attributable to the significantly larger reduction potentials of their radical cations. The acyclic hydrocarbon lycopene displayed by far the greatest activity, and one might therefore predict $lycopene^{\bullet+}$ to have the lowest reduction potential of all the carotenoids tested in this study. For reference, the reduction potential of $ABTS^{\bullet+}$ versus the standard hydrogen electrode was reported as 680 mV[62] and one might therefore expect the radical cation of a carotenoid donating an electron to $ABTS^{\bullet+}$ to have a potential lower than this under equivalent conditions.

(iii) equal rates, however, are observed for the reaction of trichloromethyl peroxyl radical ($CCl_3OO^{\bullet}$) with a range of carotenes and xanthophylls,[34] with a ranking of reactivity of β-*carotene* = *zeaxanthin* = *canthaxanthin* through a mixture of radical addition and radical cation formation; *astaxanthin* reacts more slowly solely by radical addition, as mentioned previously.

Substitution of hydrogen by carbonyl groups at the 4 and 4′-positions reduces the unpaired electron density across the 11-double-bonded carbon skeleton, resulting in a decreased propensity for electron donation, as indicated by the increase in one-electron reduction potential. This offers an explanation for the lack of reactivity of $ABTS^{\bullet+}$ with astaxanthin and canthaxanthin.[27] (A similar but less pronounced effect may also explain the lower reactivities with echinenone). This is not the case with -OH substitution at the

TABLE II Antioxidant activities of carotenes and xanthophylls relative to the antioxidant vitamins[60]

Carotenes	TEAC (mM)	Xanthophylls	TEAC (mM)
lycopene	2.9 ± 0.15	β-cryptoxanthin	2.0 ± 0.02
β-carotene	1.9 ± 0.1	zeaxanthin	1.4 ± 0.04
α-carotene	1.3 ± 0.04	lutein	1.5 ± 0.1
		echinenone	0.7 ± 0.2
Vitamins		astaxanthin	0.03 ± 0.03
vitamin E	1.0	canthaxanthin	0.02 ± 0.02
vitamin C	1.0		

3- or the 3 and 3'-positions as in β-cryptoxanthin and zeaxanthin respectively. The fact that astaxanthin and canthaxanthin are the most effective in inhibiting lipid peroxidation may relate to the alterations in electron distribution described above and to their greater propensity to participate in radical-addition pathways such as those which occur with with peroxyl radicals; the requirement for electron transfer mechanisms in the case of $ABTS^{\bullet+}$ radicals might explain the ready reactivity of $ABTS^{\bullet+}$ with the electron-dense structures of carotenes and its lack of reactivity with the ketoxanthophylls which exhibit greater electron-delocalisation across the polyene chain.

IV Sequence of Degradation of Carotenoids

Comparison of the sequence of degradation of carotenoids as they exert their antioxidant actions in a variety of biological systems *in vitro* reveals remarkably consistent results. In LDL exposed to *ex vivo* oxidation[63] promoted by Cu^{2+}, the extent of consumption after 1h oxidation is: *lycopene* > β-*cryptoxanthin* > *lutein/zeaxanthin* > α,β-*carotene*. Studies involving model lipid systems and peroxyl radical initiators under a variety of conditions do not deflect widely from this, with a sequence of *lycopene* >> β-*carotene* > β-*cryptoxanthin* ≈ *zeaxanthin* for liposomes interacting with AIBN;[64] the sequence β-*carotene* ≈ *zeaxanthin* > *astaxanthin* ≈ *canthaxanthin* for methyl linoleate/AMVN systems;[27] and the sequence *zeaxanthin* > β-*carotene* > *canthaxanthin* > *astaxanthin* for methyl linoleate and the peroxyl radical initiator AIBN.[52]

A similar sequence of carotenoid degradation (*ie.* oxidation) rates is observed during their direct interaction with the $ABTS^{\bullet+}$ radical cation, as opposed to the lipid peroxyl radicals generated in the systems described above: *lycopene* > β-*carotene* > *lutein* >> *canthaxanthin* = *astaxanthin* [Sampson, Miller, Bramley and Rice-Evans, unpublished observations]. These studies all reveal that canthaxanthin and astaxanthin degrade more slowly than β-carotene, lycopene or zeaxanthin, for example. Substituting a keto group at the 4 and 4'-position increases the efficiency of peroxyl radical trapping (and yet decreases the propensity to scavenge radicals by electron-donation), while the rates of consumption of canthaxanthin and astaxanthin are very much less than the other carotenes and xanthophylls.

V Reactivity of Carotenes and Xanthophylls with Molecular Oxygen

The reactivity of carotenoids and carotenoid-derived radicals with molecular oxygen will influence their antioxidant properties. In order to investigate the effect of carotenoid structure on degradation by oxygen, the oxidative degradation of selected carotenoids has been studied as a function of time. An acetone solution containing lycopene, β-carotene, β-cryptoxanthin and canthaxanthin, each at 1μM, was incubated under air at ambient temperature. At intervals, samples were removed from the reaction mixture and carotenoid degradation was assessed by HPLC. After 1h, the sequence for extent of loss of carotenoid was: β-*cryptoxanthin* > β-*carotene* > *lycopene* ≈ *canthaxanthin*, lycopene being one of the least degraded by oxidation during this time scale. After 6h, both the carotenes having demonstrably increased their rate of degradation relative to the xanthophylls, the sequence for extent of consumption was: *lycopene* ≈ β-*carotene* > β-*cryptoxanthin* ≈ *canthaxanthin*. After prolonged oxidation, the sequence was unchanged in that the carotenes were totally oxidised while the xanthophylls continued their progressive decline at a slower rate (Sampson, Bramley and Rice-Evans, unpublished). Since the degradation of carotenoids by oxygen is known to be an autoxidative process,[51] this may mean that xanthophylls and carotenes are approximately equally reactive in the initial reaction with oxygen, but carotene peroxyl radicals propagate more readily.

The oxidative degradation of carotenoids in solution was compared with autoxidation of the

same carotenoids within low density lipoproteins (LDL) from normal individuals. LDL samples were incubated at a concentration of 1 mg LDL protein per ml in phosphate-buffered saline containing 0.1 mM EDTA, under the same conditions of temperature and oxygenation given for the previous experiment. Carotenoids in these samples [n = 5] were present at the following concentrations: lycopene 0.35 ± 0.18, α + β-carotene 0.15 ± 0.12, β-cryptoxanthin 0.10 ± 0.02, and canthaxanthin 0.05 ± 0.08 nmol/mg LDL protein. After 1h, the sequence for percentage carotenoid loss by oxidation was: β-*cryptoxanthin* > α + β-*carotene* > *canthaxanthin* > *lycopene* (similar to that in homogeneous solution). At 6h, the sequence had changed to: β-*cryptoxanthin* ≈ *lycopene* ≈ α + β-*carotene* > *canthaxanthin*, and with prolonged oxidation: α + β-*carotene* > *lycopene* ≈ β-*cryptoxanthin* > *canthaxanthin*. Thus, within the LDL particle, lycopene is the most resistant to autoxidation on the shorter time scale, but during prolonged time periods in the presence of oxygen the carotenes are oxidised to a greater extent than the xanthophylls. The findings at 1h contrast with those after one hour's copper-mediated oxidation of LDL, where the observed sequence of consumption is *lycopene* > β-*cryptoxanthin* > α + β-*carotene*,[63] and azo initiator (AAPH)-induced LDL oxidation where lycopene is degraded faster than β-carotene at all time points (Holloway, Sampson, Bramley and Rice-Evans, unpublished). The relative position of lycopene in these hierarchies suggests that the rate of degradation of carotenoids is influenced by the addition and nature of the pro-oxidant stimulus.

The findings in homogeneous solution reported here are consistent with those of Ramakrishnan and Francis[65] who studied the relationship between the polarity of carotenoids and their relative oxidation susceptibilities over many hours. Their results showed that while the order of relative polarity increases in the order of β-*carotene*, β-*cryptoxanthin*, *canthaxanthin*, the susceptibility to oxidation by molecular oxygen decreases in the same order. They ascribed this to the oxidisability of the β-ionone ring and its stabilisation by substitutions with polar groups. The same oxidation sequence was observed here when the carotenoids are located within the LDL particle, exposed to air over prolonged timescales. Indeed, this mirrors our general conclusions concerning the relative reactivity of carotenes and xanthophylls with one-electron oxidants.

VI *In Vivo/Ex Vivo* Studies of Human LDL

A number of human studies have investigated the enhancement of the resistance of LDL to oxidation by supplementary β-carotene. In the LDL particle, the carotenoids are likely to be localised within the inner core of cholesterol esters, in contrast to α-tocopherol which is located in the outer phospholipid monolayer.[63] Considerable evidence supports a role for α-tocopherol in protecting LDL from oxidation, a process implicated in the pathogenesis of coronary heart disease. Studies have shown that a strong predictor for individuals having LDL with increased susceptibility (or lowered resistance) to oxidation appears to be a decreased vitamin E:cholesterol ratio.[66] A large range of human supplementation studies in normal populations show that supplemental vitamin E protects LDL against oxidative modification at levels ranging from 40mg (60 IU)/day to 1600mg/day.[67–72] Others have shown similar results in patients with hyperlipidaemia. Recent results have proposed that supplementation of at least 400 IU are required to decrease the susceptibility of LDL to oxidation. However, β-carotene does not apparently enhance the oxidation resistance of LDL on supplementation,[73,74] although others have shown effectiveness in smokers.[75] Furthermore, combination supplementation, including α-tocopherol and β-carotene, apparently supports the contention that only vitamin E contributes to enhancing the resistance of LDL to oxidation.[76–79] Thus, unlike α-tocopherol-enriched LDL, β-carotene-enriched LDL, produced through *in vivo* supplementation, does not dis-

play increased protection from oxidation mediated by Cu^{2+} *ex vivo*.[73,74] Is this because, in comparison with the equivalent enrichment with α-tocopherol, the enhancement of β-carotene content has not been extensive enough to reveal such protective effects? For example, an increase of LDL β-carotene level from 0.29 → 2.47 nmol/mg LDL protein or 0.25 → 5 nmol/mg protein (Table III) may not be not adequate to influence the lag phase to oxidation of LDL, which defines its oxidation resistance. The latter is also apparently dependent on the pro-oxidant applied and its concentration.[80] For example, in a study where the concentration of α-tocopherol was increased from 13.1 to 25.6 nmol/mg LDL protein by *in vivo* supplementation with vitamin E, the lag phase was prolonged much less in LDL samples oxidised by 1.0 or 1.6μM Cu^{2+} than by 0.5μM Cu^{2+}. Thus, the fact that, in contrast with α-tocopherol supplementation, β-carotene supplementation *in vivo* does not enhance the *ex vivo* resistance of LDL to oxidation as applied in these studies, might be readily explicable in terms of the absolute level of increased concentration of the β-carotene in the LDL particle, as well as the nature of the pro-oxidant applied to study the *ex vivo* susceptibility to oxidation. However, Gaziano *et al.*[73] did also apply a hydrophilic azo initiator to induce oxidation, and it is unclear, as yet, what influence varying their concentrations and conditions might have on the lag-phase to oxidation.

Alternatively, it may be that β-carotene functions very differently *in vivo* and its antioxidant properties in LDL may be less relevant. The original studies of Burton and Ingold[21] suggested that β-carotene was more efficacious as an antioxidant at low oxygen tensions, but recent studies from the group of Frei have found no protective effect of β-carotene on Cu^{2+}-induced oxidation of low density lipoproteins whether at 150 or 15 Torr.[73]

VII Human supplementation studies with β-carotene in health and disease

In an attempt to delineate the contribution of carotenoids to the protective effects of dietary fruit and vegetables, several human supplementation and intervention studies have been initiated. Table IV shows a summary of recent human studies involving supplemental β-carotene and their outcomes, revealing mixed results. The Physicians' Health study[8] finding that β-carotene has no significant benefit on cancer or cardiovascular disease in well-nourished populations has been seen in two other trials of high risk subjects, the ATBC[6] and the CARET[7]

TABLE III β-carotene supplementation and the resistance of LDL to oxidation

Supplements	duration	study group	outcome	Reference
60mg/day [Phase 1]	3 months	5f, 3m healthy nonsmokers	plasma β-C: 0.75 → 9.4μM. LDL β-C: 0.25 → 5 (nmoles/mg protein) No change in resistance to oxidation.	79
100mg/day + 50–100mg alternate days [synthetic or natural]	1 week + 3 weeks	12f, 4m healthy	plasma β-C: 0.25 → 1.39μM LDL β-C: 0.29 → 2.47 (nmoles/mg protein) No change in resistance to oxidation.	73
β-carotene 30mg/day + vitamin C 1g/day + vitamin E 800IU/day or vitamin E alone	3 months	Groups of 12 male subjects	No difference in lag phase from vitamin E alone group.	77

TABLE IV β-Carotene and combined nutrient supplementation studies in human populations

Reference	Study	Duration, dose, subjects	Outcome
81	Occurrence of new cancers in patients with previous non-melanoma skin cancer	5yr: 50mg/day	no effect
82	Oral leukoplakia prevention—preliminary study	3 months: 30mg/day	71% positive response
83	Linxian study—cancer mortality in a "healthy" population with low intake of vitamins/minerals	5.25yr: 15mg β-carotene, 30mg α-tocopherol, 50μg Se/day. 29,584 subjects.	significant decrease in stomach cancer mortality (adenocarcinoma).
84	Incidence of adenomas in normal individuals	5yr: 1g vitamin C; 25mg β-carotene; 400mg vitamin E/day. 864 subjects.	no association.
85	Indices of colonic cell proliferation; accumulation of β-carotene in colonic mucosa—in individuals with history of colonic polyps or with prior colon cancer.	3 months: 30mg β-carotene/day or placebo.	no differences on either count.
ATBC-6	Effects of supplementation on incidence of lung cancer in long-term, heavy smokers, 35 years, 20 cigarettes/day.	5–8yr: 20mg β-carotene, 50mg vitamin E/day. 19,500 subjects.	increased incidence of lung cancer among men receiving supplementation.
CARET-7	Can oral administration of β-carotene and vitamin A decrease the incidence of lung cancer in high risk populations?	3 yr: 30mg/day β-carotene + 25000 IU/day vitamin A. 11,000 smokers, 4000 asbestos exposed workers. Mean pack years 49.	Stopped prematurely due to increased incidence of lung cancer.
Physicians Health Study-8	Effect of supplementation on coronary heart disease in individuals with previous cardiac events.	12 yr: 50mg β-carotene alternate days. 22,000 male subjects.	No effect.

trials. It is of interest to note that in the ATBC study,[6] those subjects smoking *less than* 20 cigarettes per day apparently had no increased risk of disease when given β-carotene. The CARET study[7] consisted of high risk populations of smokers and asbestos workers, supplemented with vitamin A given at 7.5 times the RDA level as well as β-carotene; the effect this might have had on the results obtained should be assessed. The Linxian Study[71] in poorly nourished populations suggests that a combination of antioxidants might be more effective than a single preparation.

Out of all this wealth of evidence, the current indications are unclear as to whether β-carotene is a key constituent which has an impact on cancer incidence or mortality. It is, however, of interest to note a study demonstrating that purified β-carotene supplements produce a greater response in terms of plasma uptake than similar quantities of carotenoids (30mg) from food sources.[86] A range of supplementation studies with purified supplements has shown enhanced plasma levels to *ca.* 3.5μM,[87–90] although one study involving 90mg β-carotene supplements achieved plasma levels of 6.5μM. In contrast, supplementation with β-carotene and lycopene from fruit and vegetable concentrate for 4 weeks showed no significant effect on plasma lycopene levels.[90] Other uptake studies revealed no increase in serum lycopene concentration from unprocessed tomato juice over 4 days, whereas increased lycopene levels were found in the blood from processed tomato juice.[91] Recent studies have described the effects of lycopene-rich tomato consumption and the inverse correlation between prostate cancer and consumption of tomato sauce and pizza.[92]

The studies presented here summarise the efficacy of carotenoids as antioxidants and free radical scavengers *in vitro* and their mechanisms of action. There is little evidence for justifying an extrapolation of these findings to the *in vivo* situation. It is still not clear if carotenoids are exerting their purported health-protective effects *in vivo* through their antioxidant properties. It may be that a diet rich in high-carotenoid containing fruit and vegetables is more efficacious than individual supplements because it represents a regular, lower intake of several constituents with several mechanisms of action, as opposed to a high intake of one constituent with limited functions.

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